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Conservation of the Duchenne Muscular Dystrophy Gene in Mice and Humans

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A portion of the Duchenne muscular dystrophy (DMD) gene transcript from human fetal skeletal muscle and mouse adult heart was sequenced, representing approximately 25 percent of the total, 14-kb DMD transcript. The nucleic acid and predicted amino acid sequences from the two species are nearly 90 percent homologous. The amino acid sequence that is predicted from this portion of the DMD gene indicates that the protein product might serve a structural role in muscle, but the abundance and tissue distribution of the messenger RNA suggests that the DMD protein is not nebulin.

DUCHENNE MUSCULAR DYSTROPHY (DMD) is an X-linked, recessive, human genetic disorder that affects approximately 1 in every 3500 males in all populations that have been studied (*1*). Clinical onset of Duchenne muscular dystrophy is first observed in affected children when they are about 2 to 3 years old, the first evidence being proximal muscle weakness. Biochemical onset of this disease occurs much earlier as seen by histological examination of affected fetal muscle (*2*). Target tissue differences are also observed with regard to clinical versus histological phenotype. Specifically, heart function is only minimally affected, even though heart muscle appears very similar histologically to affected skeletal muscle (*3*). The rapid and progressive wasting of striated muscle, characteristic of DMD, leads to death by the end of the second decade.

Despite nearly 100 years of intensive study, little is known about the primary biochemical defect responsible for the destruction of muscle in individuals with DMD. We have previously described the isolation of genomic DNA that is closely linked to the DMD gene and the delineation of two small putative exons of the DMD gene within the cloned genomic regions (*4–7*). One of these exons was used to isolate a 1-kb segment of human fetal skeletal muscle complementary DNA (cDNA) (FSM 5-1) that spans a portion of the Xp21 DMD locus (*6*). We now report the isolation and characterization of cDNAs corresponding to the homologous locus in the mouse, a comparison of the human and mouse DMD gene expression patterns, and the DNA and predicted protein sequence for about 25% of the total DMD transcript.

Previous work on the identification of human DMD coding sequences was based primarily on the high degree of conservation of two small putative exons from mouse and man (*6*). Since the expression pattern of the 14-kb human DMD gene transcript (*8*) had been studied only in fetal tissues, and in view of the difficulties in obtaining large amounts of various human tissues, we extended our transcriptional studies by using the human cDNA as a probe for Northern blots of mouse tissue RNA (*9*). The human cDNA detected a 14-kb mouse mRNA species, which was present in very low levels in tissue extracts of mouse newborn leg and gravid (15-day) combined uterus and placenta and in higher levels in newborn heart, adult heart, and adult skeletal muscle (Fig. 1). The abundance of the mDMD (mouse Duchenne muscular dystrophy) transcript is roughly one-thousandth that of the mouse α tubulin transcript (Fig. 1). We detected DMD transcription in all of the striated muscle that we tested except for fetal mouse tissues containing skeletal muscle. Our inability to detect fetal transcription was most likely due to the heterogeneity of the fetal mouse tissues, the lack of differentiation in the muscle fibers at early stages of mouse development, or both (*10*).

The smooth muscle cell layers of gravid mammalian uteri undergo massive hypertrophy in preparation for parturition. The gravid uteri of E15-bearing mice (3 days before parturition) are, therefore, an excellent source of mitotically and metabolically active smooth muscle. Our finding of a very low level of mouse DMD gene transcription in E15 combined uterus and placenta is

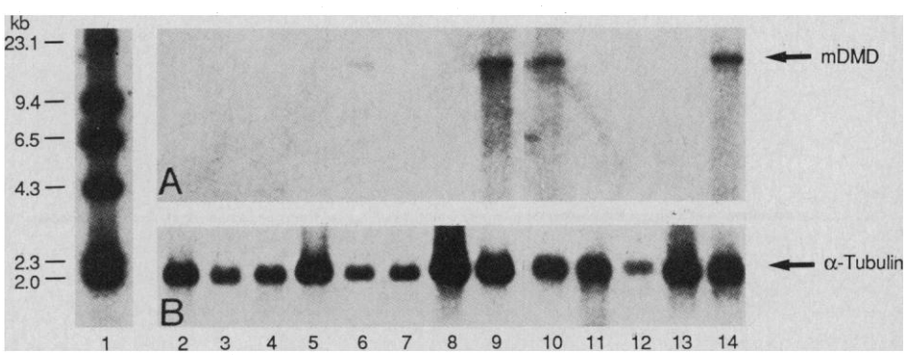

Fig. 1. Expression of the mouse DMD locus. Polyadenylated RNA (7 μg) was separated by electrophoresis in a 1% agarose-formaldehyde gel. The fragments were transferred to a nylon membrane and hybridized with a ^{32}P-labeled (*23*) human fetal skeletal muscle cDNA, corresponding to the human DMD locus (*7, 9*). The 14-kb mRNA species, corresponding to the mouse DMD gene is shown. As a control for the amount and efficacy of the RNA that was loaded, the nylon membranes were dehybridized and rehybridized with a labeled cDNA clone, corresponding to the mouse α-tubulin gene. The 2-kb mRNA, corresponding to α-tubulin, is shown for each lane. In (**A**) (mDMD) autoradiographic exposure was for 10 days; in (**B**) (tubulin) exposure was for 2 hours, that is, one-hundredth the time used for (A). All autoradiography was done with an intensifying screen at −80°C, and each probe was labeled to similar specific activities. The same blots used with the human cDNA probe were then dehybridized and again probed with the mouse cDNA, MC2-6. The results with the mouse probe were identical to those with the human probe. Lanes are as follows: 1, λ DNA Hind III–digest markers; 2, E13 (13-day-old embryos); 3, E13 uterus and placenta; 4, E15 carcass; 5, E15 brain; 6, E15 uterus and placenta; 7, E15 viscera; 8, newborn brain; 9, newborn heart and lung; 10, adult heart; 11, adult kidney; 12, adult liver; 13, adult lung; 14, adult skeletal muscle. Additional tissues that were tested but not shown were as follows: mouse gravid uteri of embryonic day 5 (E5), E7, and E9; isolated mouse embryos of E10 and E11; adult brain, spleen, small intestine, stomach, and testes. All of these tissues tested negative for the mDMD transcript.

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most likely due to expression in the abundant smooth muscle tissue. Thus, the expression pattern of the human DMD gene in mouse is identical to, and otherwise complements, the expression pattern of the human DMD in fetal tissues (*7, 11*).

In order to obtain cDNAs representative of the mouse DMD gene, a cDNA library was constructed from adult mouse heart (*12*) and screened with the partial cDNA corresponding to the human DMD gene (FSM-5). Seven mouse cDNA clones were obtained from 5×10^5 primary (unamplified) λ gt10 recombinants. Six of these were encompassed within the largest 2.7-kb cDNA, MC2-6, corresponding to the sequences shown 5′ to the Eco RI site in Fig. 2. The additional 1.7-kb of cardiac cDNA sequences, 3′ of the Eco RI site, were isolated from the same cDNA library by screening with a mouse skeletal muscle cDNA clone that had been isolated from an adult skeletal muscle cDNA library. All of the clones that have been isolated from the mouse skeletal muscle library to date are identical to those isolated from the mouse cardiac muscle library. In this second screening of the cardiac muscle library ten identical clones were obtained.

The low frequency of DMD clones obtained in both human (*6*) and mouse cDNA libraries suggests that the DMD messenger RNA (mRNA) is rare, probably representing about 0.01 to 0.001% of the mRNA in muscle. This relative abundance is in agreement with our Northern blot analyses and the frequency with which recombinant DMD clones were obtained from four other mouse and human cDNA libraries constructed in our laboratory.

Fragments of the mouse cDNA were then used as probes for Southern blots of mouse genomic DNA. At least 12 X chromosome–specific, Hind III–digested DNA fragments were detected by the 5′ fragment (2.7 kb) of cDNA sequences (MC2-6). Ten are evident in Fig. 2 (*13*). Since there was only one internal Hind III site in the mouse cDNA clone, this 2.7-kb clone hybridized to a minimum of 11 regions distributed over at least 90 kb of the mouse X chromosome (*13*). In view of the 14-kb size of the complete mRNA, and if we assume a constant ratio of cDNA to genomic DNA, the complete mouse DMD genomic locus probably encompasses more than 500 kb of genomic DNA. Thus, the hybridization characteristics of the X-linked human DMD (*7, 8*) are also conserved in the mouse.

The human fetal skeletal muscle cDNA library, originally used to obtain the 1-kb FSM 5-1 cDNA (*6*), was completely digested at Eco RI sites because methylation did not occur at those sites during construction of the cDNA library. Since the 3′ terminus of FSM 5-1 contained an endogenous Eco RI site rather than an added linker, we used a genomic exon fragment spanning the Eco RI site as a probe to obtain cDNAs on the 3′ side of this Eco RI site. In this manner we obtained a 1.2-kb cDNA fragment that was contiguous with that described previously. Additional Hind III–digested, X chromosome–specific, genomic DNA fragments, were detected by this cDNA (Fig. 2).

The DNA sequences of the human and mouse DMD cDNAs were determined throughout the region of overlap (2.2 kb), with the mouse sequence extending approximately 500 bp both 5′ and 3′ of the overlap (Fig. 3). The total nucleotide sequence of 3.3 kb represents nearly 25% of the entire DMD mRNA and contains one continuous open reading frame. Both the DNA and amino acid sequences are well conserved, exhibiting 88% homology with the DNA and 87% with the amino acid sequence. There is a particularly striking conservation in the hydropathicity profile of the mouse and human amino acid sequences (*14*), and the hydropathicity profiles are nearly identical (Fig. 4). Indeed, if conservative amino acid substitutions are permitted based on hydropathicity values, the mouse and human polypeptides become more than 95% homologous.

The protein sequence that is predicted from this portion of the DMD gene has a number of interesting characteristics. Chou and Fasman calculations predict a strong propensity for an α-helical secondary structure over the entire 100 kD of protein sequence (*14*). Locally, charged residues alternate with hydrophobic residues in a manner resembling those found in tropomyosin and the myosin rod, both which contain hydrophobic residues concentrated at two positions within a heptad repeat. This creates a hydrophobic interface between neighboring α-helical coils, resulting in a coiled-coil (*15*). In fact, NBRF protein database searches found homologies to many such α helix–rich, coiled-coil proteins (*14*). For example, regions of more than 100 amino

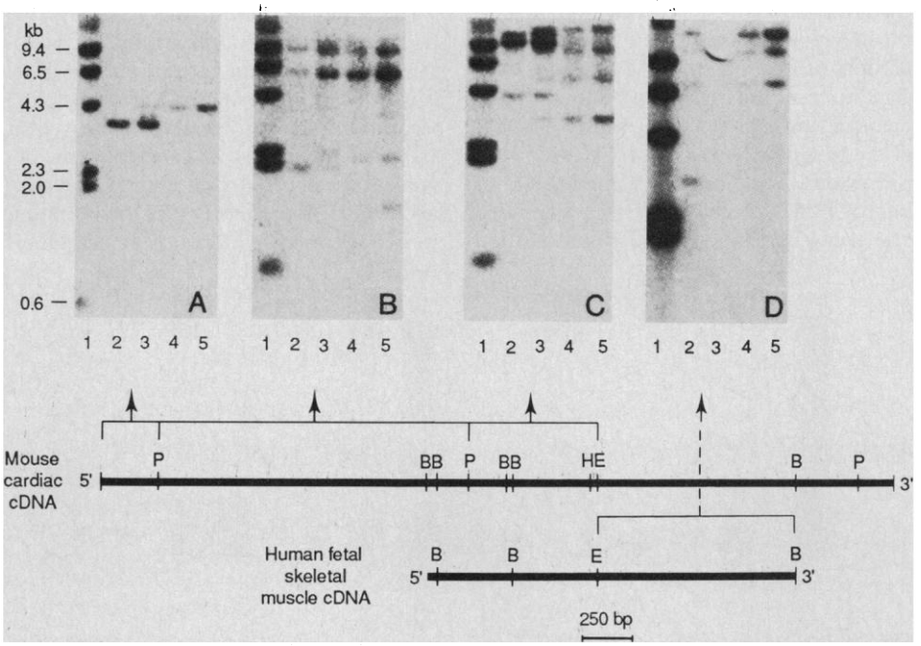

Fig. 2. Restriction maps and genomic analyses of partial DMD cDNAs in mouse and man. This is an extension of a previously described human fetal skeletal muscle DMD cDNA segment [FSM-5; (*7*)] and the overlapping mouse adult heart cDNA segments. The human cDNA library has been described (*7*). The mouse adult heart cDNA library was constructed in λ gt10 (*13*). DMD-homologous sequences from both human and mouse recombinant phage were subcloned into plasmid vectors (Bluescript, Stratagene) and used as probes against Southern blots of genomic DNA (*23, 24*). A total of 10 of the 12 mouse genomic Hind III fragments recognized by the mouse cDNA, MC2-6 (**A**, **B**, and **C**) (*13*), are shown in addition to the human genomic Hind III fragments recognized by the extension of the human cDNA (**D**). Because of the high degree of conservation of this locus (*7*), all of the cDNA clones exhibited an X chromosome–specific pattern and recognized genomic fragments in the hamster DNA backgrounds. Lanes for panels A, B, and C are the following Hind III–digested genomic DNA samples: 1, labeled (^{32}P) λ DNA Hind III digest markers; 2, hamster; 3, hamster somatic cell-hybrid bearing a mouse X chromosome; 4, male mouse; and 5, female mouse. Panel D has the following Hind III–digested DNA samples: 1, radiolabeled λ DNA Hind III digest markers; 2, hamster hybrid cell line containing a human X chromosome; 3, lymphoblastoid cell line from a patient having an Xp21 deletion (*25*); 4, normal male, 46,XY; 5, 49,XXXXY cell line (GM1202). Restriction enzyme sites are as follows: P, Pst I; E, Eco RI; H, Hind III; and B, Bgl II.

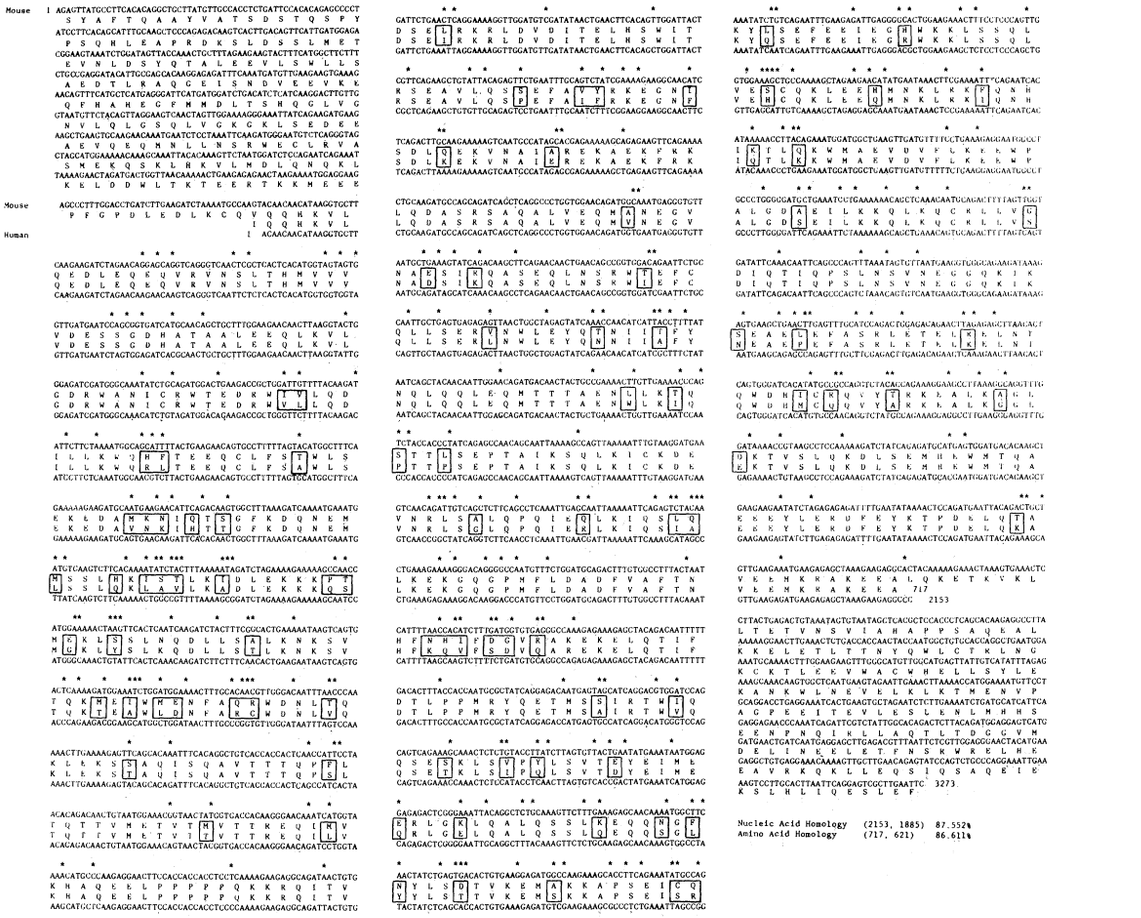

Fig. 3. Nucleotide and predicted amino acid sequences of mouse and human cDNAs for a portion of the DMD gene. The cDNAs shown in Fig. 2 were subcloned in plasmid vectors (Stratagene Bluescript) and subjected to either chemical (*26*) or chain terminating (*27*) sequencing methods. Clones were sequenced on both strands. Sequence translations and alignments were done on the BIONET resource, Intelligenetics. Differences between the mouse and human DNA sequences are indicated by asterisks, while amino acid differences are boxed.

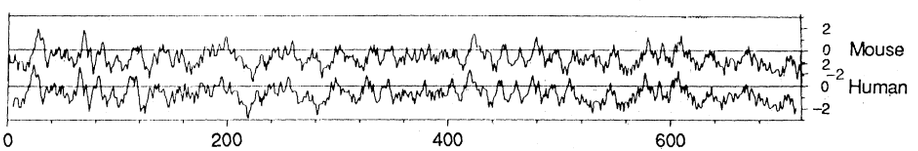

Fig. 4. Hydropathicity profiles of sequenced regions of the DMD cDNAs. Hydropathicity plots, obtained with Kyte and Doolittle calculations (*14*), were done at the Howard Hughes Medical Institute Computing Center, Harvard Medical School, with the use of default values of the University of Wisconsin's PEPPLOT program. The horizontal axis shows the amino acid residue number of the human sequence; the vertical axis gives the relative hydropathicity of each plot.

acids of the DMD protein are 20% homologous to the myosin rod of both nematode and rat (*14*). Unlike these perfect coiled-coils, however, this section of the DMD protein contains occasional helix-breaking residues, suggesting that it might adopt an α-helical bundle conformation, rather than an extended rod structure (*16*). In addition, the initial 650 residues exhibit a fairly constant ratio of charged to apolar amino acids of 0.7, whereas extended rod structural proteins usually have ratios from 0.9 to 1.4 (*16*). In the NH_2-terminal fragment, containing 440 residues, the ratio of charged to apolar amino acids is 1.0, and there are fewer prolines and a stricter adherence to the heptad repeat.

The high degree of homology in the sequence of this protein from mouse and man suggests either that it interacts with a number of other highly conserved structural proteins or it serves a specific structural role in muscle tissue. The low ratio of charged to apolar amino acid residues in the NH_2-terminal region suggests that the protein product of the DMD gene might be associated peripherally or integrally with the muscle fiber plasma membrane (sarcolemma). However, the primary amino acid sequence is also reminiscent of most sarcomeric proteins, such that a structural role for the DMD product within the sarcomere is also possible.

A large sarcomeric protein, nebulin, has been proposed as a candidate for the DMD gene product due to its large size (500 kD) and its apparent absence in the muscle of affected individuals (*17*). Nebulin has been estimated to represent roughly 3% of total myofibrillar protein (*18*). We have calculated that 0.01 to 0.001% of total cardiac and skeletal muscle mRNA is DMD mRNA. Although it is difficult to equate the abundance of a particular protein to the abundance of its corresponding mRNA, this large difference between the amounts of nebulin protein and DMD mRNA seems significant. Comparative studies of the distribution of nebulin throughout the animal kingdom have revealed an analogue of nebulin in the skeletal muscle of most organisms but not in any cardiac muscle (rabbit, sheep, cow, rat, and bullfrog) (*19, 20*). We have shown that the DMD gene is expressed equally well in skeletal and cardiac muscle in both humans and mice (Fig. 1). The differences in tissue distribution and abundance between the DMD mRNA and the nebulin protein suggest that nebulin is probably not the primary product of the DMD gene. However, the disturbed patterns of nebulin protein homeostasis observed in DMD-affected individuals implicate nebulin as a potentially important component in the etiology of this disease.

The prominent abnormality in boys affected with DMD is the loss of skeletal muscle. Also associated with DMD are car-

diac abnormalities (*3, 21*) and, less often, mental retardation (*22*). The transcriptional data that have been presented for the DMD gene thus far account for the skeletal and cardiac muscle defects, but do not explain the mental retardation. When more is understood about the DMD and surrounding Xp21 loci, the mental retardation seen in some DMD boys may be explained. Further insights into the basic biochemical defect in DMD must await specific protein studies with antibodies directed against the DMD protein product, analyses of the specific mutations giving rise to the DMD phenotype, and cell-based expression studies.

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8. The size of the DMD transcript has been lowered to 14 kb due to our recent cloning of the entire DMD human cDNA [M. Koenig *et al.*, *Cell* **50**, 509 (1987)].
9. RNA was isolated from tissues by homogenization of frozen, ground tissues in guanidium thiocyanate, followed by pelleting through a CsCl cushion [J. M. Chirgwin, A. E. Przybla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **18**, 5294 (1979)]. Polyadenylated RNA [H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408 (1972)] was separated by electrophoresis in 1% agarose formaldehyde gels and then transferred to nylon membranes. Hybridization was performed as recommended by the manufacturer [Biodyne A, New England Nuclear; P. S. Thomas, *Proc. Natl. Acad. Sci. U.S.A.* 77, 5201 (1980)].
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11. The number and positions of the RNA samples on the Northern blots that were reported previously (*6*) were incorrectly assigned. The human DMD gene transcription attributed to expression in small intestine and lung was instead due to expression in human fetal heart. Later experiments have shown that the expected 14-kb human DMD transcript is present in fetal heart at levels equivalent to those found in fetal skeletal muscle.
12. The cDNA library was constructed using a modification of the oligo(dT) primed, ribonuclease H procedure of U. Gubler and B. J. Hoffman [*Gene* **25**, 263 (1983)], with modifications suggested by Stratagene's librarian, H. Short, including a Sepharose 4B exclusion column to eliminate Eco RI linkers and cDNA molecules shorter than 600 bp. We used the vector λ gt10 [T. V. Huynh, R. A. Young, R. W. Davis, in *DNA Cloning*, D. M. Glover, Ed. (IRL Press, Washington, DC, 1985), vol. 1, pp. 49–78].
13. Mouse genomic locus size was calculated as follows; mouse genomic DNA, digested with Hind III, was fractionated on a 10-cm, 0.7% agarose gel, transferred to nitrocellulose (*24*) and hybridized with five overlapping subfragments of MC2-6 (Fig. 2). Two additional, weakly hybridizing Hind III fragments were observed but are not evident in this figure. The single-copy genomic fragments detected by the five probes are 22 kb, 12 kb, 11 kb, 8.5 kb, 8 kb, 5.9 kb, 5 kb, 4.9 kb, 4.3 kb, 3 kb, 2.1 kb, and 1.1 kb. These fragments total more than 90 kb and represent a minimum estimate of the genomic size as there are probably many more Hind III genomic DNA fragments lying between those detected by our probes (*6*).
14. Hydropathy measurements and plots, protein secondary structure predictions, NBRF (National Biomedical Research Foundation) protein sequence database searches, and lowered stringency homology searches between myosin and the DMD protein were done on the BIONET (Palo Alto, CA) or the Howard Hughes Medical Institute (Harvard Medical School, Boston, MA) computer systems. Both the University of Wisconsin Genetics Computer Group (U-WISC) and Intelligenetics (BIONET) software packages were used. α helix–β sheet propensities were calculated according to P. Y. Chou and G. D. Fasman [*Biochemistry* **13**, 222 (1974)]. Hydropathy measurements were calculated according to Kyte and Doolittle [*J. Mol. Biol.* **157**, 105 (1982)]. Figure 4 shows the hydropathy plot output of the U-WISC PEPPLOT program.
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28. We thank H. Short for her cDNA library protocol, S. P. Kwan and S. Whitehead for the generous synthesis of oligonucleotides, D. Shine for his gift of a mouse α-tubulin cDNA clone, C. Disteche and R. Farber for their gift of the hamster somatic cell hybrid bearing a mouse X chromosome, R. Neve for supplying a human fetal Northern blot and skeletal muscle cDNA library, and J. Schwaber, S. Latt, and C. Cohen for helpful criticisms of the manuscript. E.H. is the Harry Zimmerman Post-doctoral Fellow of the Muscular Dystrophy Association. A.P.M. is supported by the Muscular Dystrophy Association and PHS NRSA (2T 32 GM07753-07) from the National Institute for General Medical Sciences. Supported by grants to L.M.K. from the Muscular Dystrophy Association and the National Institutes of Health (R01 NS23740 and HD18658). L.M.K. is an associate investigator of the Howard Hughes Medical Institute.

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Depolarization Without Calcium Can Release γ-Aminobutyric Acid from a Retinal Neuron

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Calcium influx is often an essential intermediate step for the release of neurotransmitter. However, some retinal neurons appear to release transmitter by a mechanism that does not require calcium influx. It was uncertain whether depolarization released calcium from an intracellular store or released transmitter by a mechanism that does not require calcium. The possibility that voltage, and not calcium, can regulate the release of transmitter was studied with pairs of solitary retinal neurons. Horizontal and bipolar cells were isolated from fish retinas and juxtaposed in culture. Communication between them was studied with electrophysiological methods. A horizontal cell released its neurotransmitter, γ-aminobutyric acid, when depolarized during conditions that buffered the internal calcium concentration and prohibited calcium entry. The speed and amount of material released were sufficient for a contribution to synaptic transmission.

CALCIUM-TRIGGERED EXOCYTOSIS operates to release transmitters at neuromuscular junctions, between peripheral neurons, and in the brain. Synapses that utilize this mechanism are often recognized by two features: first, an aggregation of vesicles marks each presynaptic site and second, Ca^{2+} is required for transmitter release. Exceptions to this ubiquitous mechanism may now have been identified in the distal retina (*1, 2*). Studies of its anatomy and physiology indicate that both photoreceptors and horizontal cells make synapses that operate differently.

Photoreceptors make two morphological types of synapse (*3*): one has an aggregation of vesicles at a release site, the other is vesicle poor. Isolated photoreceptors release transmitters by two mechanisms (*2*): one requires extracellular Ca^{2+}, the other continues in the absence of Ca^{2+}. Moreover, one component of the normal synaptic transmission from photoreceptors to postsynaptic cells requires extracellular Ca^{2+} (*4*), while another component functions without extracellular Ca^{2+} (*5*).

Complementary observations have been made for horizontal cells. They make synapses that lack vesicles (*6*) and release the

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